The Role Of DNA Damage Tolerance in Hematopoietic Stem Cells, Cancer Therapy, and Somatic Hypermutation

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Discussion

In this thesis I focused on the role of DNA damage tolerance (DTT). In particular, this thesis addresses anti-and pro-mutagenic aspects of the DDT system as an integral part of the DNA damage response network.

Anti-mutagenic role of PRIMPOL

In chapter three, we investigated the role of mammalian DNA damage tolerance translesion synthesis (TLS) and primase PRIMPOL (1-3). We uncovered a novel anti-mutagenic role for PRIMPOL, both in murine B cells as well as in human invasive breast cancer. PRIMPOL seems to prevent mutations instigated by abasic sites. Abasic sites are central intermediates of spontaneous or catalyzed depurination or depyrimidation reactions.

Furthermore, also in invasive breast cancer with loss of *PRIMPOL,* an increased amount of APOBEC induced mutations was found. APOBEC is a member APOBEC/AID family that catalyze the deamination of C to U in RNA or DNA.

As PRIMPOL is unable to perform TLS opposite of an abasic site under nuclear conditions, it is likely that PRIMPOL acts as an anti-mutagenic primase during bypass of abasic sites (1-4). Our model envisioned repriming by PRIMPOL prevents mutagenesis through inhibition of TLS and promoting homology directed DNA damage bypass, such as template switching. In conclusion, the error-prone TLS polymerase and primase PRIMPOL has an anti-mutagenic function in the DDT of abasic sites, which likely relates to its primase activity. Further experiments are required to unravel the molecular details of the preferential targeting of PRIMPOL and the regulation of its anti-mutagenic activity.

PRIMPOL on the leading strand

Since the first description of PRIMPOLs function in DDT, PRIMPOL has been proposed to be active on the leading strand (1-3). This hypothesis was based on the fact that POLA continuously reprimes on the lagging strand, rendering PRIMPOL unnecessary on the lagging strand. In chapter four, we reveal that the strand-biased anti-mutagenic role of PRIMPOL strongly correlates with a role or preferential activity on the leading strand in the *Igh* locus. In conclusion, PRIMPOL has an anti-mutagenic role in tolerating AID/APOBEC induced abasic sites. By acting as a primase downstream of abasic sites in the leading strand, it somehow facilitates error-free processing, presumably by taking advantage of homology directed template switching. Of note, the first in support of this function are provided in chapter 4. Another study claiming a preferential role for the tolerance of PRIMPOL on G4 stacks on the leading strand which results in epigenetic instability in absence of *PrimPol* (5) does not provide this evidence. The reasoning, their read-out only works on the leading strand as G4 structures on the lagging strand have not been found to lead to epigenetic instability (6,7). Therefore, using this readout a role for PRIMPOL on the lagging strand cannot be excluded. Furthermore, this study was performed in chicken DT40 B lymphoma cell line, and the found

epigenetic instability in response to a defect in G4 tolerance has not been corroborated in mammalian systems. In summary, while our data indeed provide first evidence for a role of PRIMPOL exclusively on the leading strand, more research needs to be performed to corroborate the central role of PRIMPOL in repriming on the leading strand.

Impairing base excision repair for effective somatic hypermutation

In chapter four, we investigated the role of base excision repair in somatic hypermutation. During SHM, AID generates deoxy-uracil (U) in the *Ig* loci (8,9). Generally, Us are very efficiently repaired by the base excision repair (BER) pathway (10). BER deals with approximately 500 Us from spontaneous deaminations and 10,000 abasic sites per cell per day and repairs these error-free (11,12). In contrast, in hypermutating B cells BER components UNG and APEX2 have been shown to be key pro-mutagenic factors (13-21). Over the last decades, the question why AID generated Us are highly mutagenic and not repaired conventionally by the BER remained unanswered. Two scenarios could explain error-prone processing, AID outcompetes canonical BER, or canonical BER is prohibited to favor non-canonical, mutagenic processing of lesions instigated by AID.

In literature, the role of the BER protein POLB regarding mutagenesis has been somewhat controversial (22,23). The potential reason of the controversy is uncovered in this chapter. We revealed that POLB, a key polymerase in short-patch BER is downregulated post-transcriptionally in germinal center B cells. POLB exerts a key function by filling in the gap during short patch BER.

In line with the second scenario, POLB degradation appeared key in promoting SHM ncMMR at template A or T. *In vitro* hypermutation systems, which are generally characterized by lack of infrequent A/T mutations, preferentially increased A/T mutagenesis upon POLB knockdown. As mentioned, A/T mutagenesis depends on non-canonical mismatch repair (ncMMR), and indeed recruitment of ncMMR components increased when POLB becomes limited. We propose that active POLB degradation allows ncMMR EXO1 to extend short single stranded gaps which are otherwise filled in by POLB and thereby facilitate error-prone non-canonical repair synthesis by the PCNA-Ub/POLH complex, known to generate the vast majority of A/T mutations. In line, APEX2 is involved in A/T mutagenesis (18,21). Alternatively, POLB downregulation leads to an increase of U-G mismatches due to impaired BER, which in turn favors ncMMR. In this context, passing the baton mechanism in the absence of POLB may lead to UNG2 getting stuck on DNA and unable to initiate BER and target new Us (24,25). This would decrease the BER activity. The increase in U-G mismatches are then targeted by ncMMR, leading to increased A/T mutagenesis. In line, C/G transitions are slightly increased upon POLB, suggesting an increase of U. In addition, we reveal that hypoxia as found in germinal centers, downregulates POLB protein, through increased POLB turnover (26). These results shed light on the long-standing

question why error-free short patch BER pathway fails to restore AID generated Us during SHM. Apparently, to optimize antibody affinity by SHM, germinal center B cells appear to take a great risk by switching transiently from error-free BER to pro-mutagenic ncMMR. Furthermore, POLB is dominant over ncMMR in the repair of either the U or gap.

PCNA K164 and Rev1 pathways of in regulation of DNA damage tolerance

In chapter five, we assess the role of REV1 and PCNA K164 in DNA damage tolerance. *Rev1* is known to have a fork progression defect upon UV irradiation, likely due to fork stability, while *Pcna*^{KR} does not lead to this defect (27-29). Furthermore, we investigate the role of PCNA K164 and REV1 in DDT through Y-family polymerase recruitment (28-36). In addition, PCNA K164 is key in fork reversal and template switching (35,37-40). In order to investigate the role of PCNA K164 and REV1 and their role in DDT, we analyzed recruitment of Y-family polymerase POLH, POLI, POLK, and REV1 through DNA damage induced foci formation analysis in immortalized MEFs. We show that upon UV exposure, POLH shows the highest number of foci, followed by REV1, POLK, and POLI. In line, POLH can tolerate cyclobutane pyrimidine dimers (CPDs) and 6-photoproduct (6-4PP) (41). REV1 can recruit itself into foci independent of PCNA-Ub. Also, a *Rev1* knock-out leads to a decrease of foci of the other tested TLS polymerases, suggesting REV1 can recruit Y-family independently of PCNA K164. Though REV1 seems at least in part independent of PCNA K164, this analysis revealed that PCNA K164 is more important for induction of foci formation than REV1. *Rev1*$^{-/-}$ MEFs also shows a decreased number of POLH and POLK foci, though not to the extent of *Pcna*^{KR}. Deficiency in both REV1 and PCNA K164 double impairment leads to the same level of foci as *Pcna*^{KR}, indicating REV1 and PCNA K164 are epistatic in the recruitment of POLH and POLK upon UV.

Interestingly, double mutant MEFs revealed an increased number of POLI foci, whereas these foci are nearly absent in single mutant and WT MEFs. In conclusion, PCNA K164 is more important in regulating survival and Y-family TLS foci formation as compared to REV1. Though, REV1 has a bigger effect on fork progression. This fork progression might be explained by a role for REV1 in preserving replication tracts form degradation, analogous to FANCD2 and BRCA1/2 (27).

Targeting DNA damage tolerance defects in cancer therapy

In chapter six, we show that many tumors have defects in genes in specific DNA damage tolerance pathways. This led to the notion that common DDT defects may be targeted using synthetic lethal approaches or DNA damaging drugs for which these tumor cells are specifically sensitive. We show that T-cell lymphoma and breast cancer specific *Pcna*^{K164R} mutation can be more effectively treated by cisplatin then their isogenic wildtype counterpart. Further research into synthetic lethal combinations should expand the possibilities of

targeting tumors with specific DNA damage tolerance defects.

DNA damage tolerance and hematopoietic stem cell aging

In chapter seven, the role of DNA damage tolerance in adult hematopoiesis was analyzed, using DDT impaired *Pcna*^{KR} mice. *Pcna*^{KR} HSC show increased aging and are impaired in both non-competitive and competitive translation assays. The aging defect in *Pcna*^{KR} mice is revealed by an increased cellularity of myeloid/erythroid multipotent progenitor 2 (MPP2) seemingly at the expanse of progressively decreasing lymphoid MPP4 and an increased level of DNA damage in LSK cells. This differentiation bias is thought to be at the base of the decreased B and T lymphocyte diversity with age. In conclusion, DNA damage tolerance maintains HSC fitness ad prevents premature ageing.

Cell cycle and sensitivity to DNA damage tolerance defect

In theory, the cell cycle of HSC and progenitor subsets should correlate to the relative decrease. While this seems true for most stem cell and progenitor subsets, the slower cycling ST-HSC and MPP4 have the largest fold change in cell number, compared from WT to *Pcna*^{KR}, which suggests that the ST-HSC and MPP4 subsets differentiation increases towards other progenitors. Next to decreased cellularity of common myeloid progenitor (CMP) and granulocyte and macrophage progenitor (GMP) subsets, we also observed increased cell cycle arrest CMP and GMP subsets in *Pcna*^{KR} mice. This effect is in line with the *Pcna*^{KR} defect and the close link to replication and suggests that CMP and GMP subsets have increased replication fork stalling and increased levels of DNA damage. Strangely, these subsets do not show increased γH2AX, a mark often used to measure the level of DNA damage. In conclusion, DNA damage tolerance plays a key role in cell cycle progression of the CMPs and GMPs. Though it remains unclear what underlies the cell cycle defect in these subsets.

DNA damage tolerance is essential for hematopoietic stem cell maintenance and mammalian life

As mentioned above, overall REV1 and PCNA K614 dependent DDT pathways have a non-epistatic relationship in regulation of DDT, as investigated in chapter 8. To research the full relevance of DDT to HSC maintenance, we made compound DDT defective *Pcna*^{KR}*;Rev1*$^{-/-}$ mice. This mouse model revealed that these DDT is essential for life, as no double mutants were born. Even though the double mutant mice were never found, we were able to isolate E18.5 embryos for analysis at sub-mendelian inheritance ratio. The *Pcna*^{KR}*;Rev1*$^{-/-}$ embryos revealed that the embryos likely perished due to an erythroid crisis due to HSC failure. In conclusion, DDT is essential for HSC maintenance and life. The results also reveal DDT is an essential part of the DNA damage response.

PCNA K164 and Rev1 pathways of DNA damage tolerance in survival

PCNA K164 monoubiquitination and REV1 can both recruit TLS polymerases (31,32,34). In addition, PCNA K164 polyubiquitination is involved in template switching and fork reversal (37,40). Deficiency in both REV1 and PCNA K164 leads to a decreased replication fork rate, both under steady state replication as well as upon exposure to ultraviolet light (UV) exposure (chapter nine). Furthermore, immortalized MEFs were exposed to DNA damaging agents UV, cisplatin, formaldehyde, and 4-hydroxynonenal (4HNE). For UV and cisplatin exposure, double mutant $Pcna^{KR};Rev1^{-/-}$ MEFs are more sensitive that single mutant and WT controls. Furthermore, $Rev1^{-/-}$ single mutants shows no clear change in survival compared to WT in response to UV or cisplatin. In the case of formaldehyde, $Pcna^{KR};Rev1^{-/-}$ is only slightly more sensitive compared to $Pcna^{KR}$. While, $Rev1^{-/-}$ is equally sensitive as WT. $Pcna^{KR};Rev1^{-/-}$ are most sensitive to 4HNE, followed by $Rev1^{-/-}$, $Pcna^{KR}$, and WT is least sensitive. In general, under genotoxic stress PCNA K164 has a bigger effect on survival than REV1. In summary, both endogenous and exogenous DNA damage contribute to PCNA K164 and REV1 dependent DTT responses.

The role of the erythroid committed progenitor (ECP)

Single cell sequencing of HSC and early progenitor LSK cells of DDT defective mice suggest the existence of a novel subset within this population. Based on transcriptome analysis, this population was identified as erythroid committed progenitor cells. Their transcriptome revealed increased erythroid lineage transcription factors as *Klf1* and *Zbtb7a* among other factors. In addition to being upregulated at the mRNA level, KLF1 transcription domain was found necessary in warranting the formation of the ECP subset. Apparently, ECPs strictly depend on KLF1. In conclusion, this novel ECP likely contributes to erythrocyte generation and is predominantly maintained upon stress.

Differentiation of MPP4 towards erythroid committed progenitor under crisis

The plasticity of cells during hematopoiesis decreases during development. The further cells differentiate, the more restricted their developmental path becomes. During ageing, the cellularity of lymphoid-biased MPP4 subset decreases steadily, seemingly at the expense of MPP2 and MPP3 (42). Similar to this ageing related process, also in $Pcna^{KR};Rev1^{-/-}$ embryos MPP4 were found to decrease most in comparison to all other LSK subsets. Clustering of single cell RNA sequencing revealed that in $Pcna^{KR};Rev1^{-/-}$ double mutant embryos a lot of MPP4 cells cluster to the ECP subset. Similarly, also correlative analyses of differential transcriptomes of different HSC and progenitor subsets, revealed a part of MPP4 cells according to flow cytometry markers (LSK, $CD135^{+}$, $CD150^{-}$) actually do not correlate to the MPP4 signature transcriptome. These MPP4 cells that have a low correlation coefficient were actually identified as ECP cells. In conclusion, these data suggest that under stress

conditions particularly MPP4 cells can differentiate into ECP cells to warrant tissue oxygenation.

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